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Generation of endogenous BMP transcriptional reporter cells through CRISPR/Cas9 genome editing

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Abstract
Transcriptional reporter systems allow researchers to investigate the function and regulation of transcription factors. Conventional systems employ artificial cDNA overexpression vectors containing either a promoter fragment or specific nucleotide sequence repeats upstream of *firefly* luciferase or fluorescent reporters, such as green fluorescence protein (GFP) cDNA (1). These systems suffer mainly from the lack of chromatin context. Here, we describe the rapid generation of endogenous transcriptional reporter cells for the Bone Morphogenetic Protein (BMP) pathway using CRISPR/Cas9 genome editing. In principle, our methodology can be applied to any cell line. The endogenous reporters will provide a robust system for the investigation of BMP transcriptional activity in the context of native chromatin landscape and facilitate chemical and genetic screens.

Introduction
Bone Morphogenetic Proteins (BMPs) are members of the Transforming Growth Factor Beta (TGFβ) superfamily of cytokines, with crucial roles in embryogenesis and adult tissue homeostasis. Aberrant BMP signaling is thus associated with numerous developmental defects and human pathologies (2). BMPs signal via binding to and activation of cognate type I and type II BMP receptor serine/threonine kinases. The activated type I BMP receptors subsequently phosphorylate the intracellular SMAD transcription factors 1, 5 and 8 at a C-terminal Ser-X-Ser motif, thereby inducing their interaction with the common mediator SMAD4. The SMAD1/5/8-SMAD4 complex is then able to translocate into the nucleus, where it associates with various transcriptional co-factors to regulate gene expression (3). Inhibitor of differentiation 1 (ID-1), a dominant negative regulator of basic helix-loop-helix (bHLH) transcription factors, is a direct gene target of BMP signaling (4). Therefore, ID-1 mRNA and protein expression is frequently employed as a downstream readout of the BMP signaling pathway.

We have previously reported the generation of endogenous transcriptional reporter cells for the TGFβ pathway using CRISPR/Cas9 genome editing (5). This system involved the insertion of a transcriptional reporter cassette downstream of the endogenous promoter for the TGFβ target gene plasminogen activator inhibitor 1 (PAI-1). In the following protocol, we describe the generation of an endogenous BMP transcriptional reporter cell line, in which a reporter cassette containing *firefly luciferase* and *GFP* gene, has been inserted adjacent to the endogenous *ID-1* promoter sequence. The generation of the cell line thus enables the study of endogenous BMP transcriptional activity as well as chemical and genetic high-throughput screens.

2. Materials

2.1 Mammalian cell culture and cell transfection reagents

1. Dulbecco's Phosphate Buffered Saline (DPBS) 1x
2. 0.05% Trypsin-EDTA
3. Dulbecco's Modified Eagle Medium (DMEM), 4.5 g L^{-1} *D*-Glucose
4. Fetal Bovine Serum (FBS)
5. Penicillin-Streptomycin (10,000 Units mL^{-1})
6. *L*-Glutamine (200 mM)
7. Opti-MEM (Reduced Serum Medium)
8. Polyethyleneimine (PEI) MAX (MW 25000)

2.2 Cytokines and inhibitors

1. Recombinant Human BMP-2 Protein
2. SB-505124 (ALK4/5/7 inhibitor)
3. LDN193189 (ALK2/3 inhibitor)
4. DMSO

2.3 Luciferase assay

1. 2x Luciferase Assay Buffer: 50 mM Tris/phosphate (p*H* 7.8), 16 mM $MgCl_2$, 2 mM DTT (Dithiothreitol), 1 mM ATP, 30% (v/v) glycerol, 1% (w/v) bovine serum albumin (BSA), 250 µM *D*-Luciferin and 8 µM sodium pyrophosphate.
2. Luciferase Cell Culture Lysis 5X Reagent (CCLR)
3. Cell Culture Microplate (96-well), Flat-Bottom, White, Sterile
4. Plateseal, Permanent Seal for Microplates
5. EnVision Multilabel Plate Reader (2104-0010A, PerkinElmer)
6. Coomassie (Bradford) Protein Assay Kit

3. Methods

3.1 Design of guide RNA (gRNA)

A dual guide RNA (gRNA) approach was undertaken with sense and antisense gRNAs designed to target a region (corresponding to nucleotides 41-84) within the *N*-terminal coding region of the *ID-1* gene.

- Sense gRNA (5'-CGGCAAGACAGCGAGCGGTGCGG-3') cloned into pBABE-Puro U6 vector containing puromycin resistance sequence
- Antisense gRNA (5'-CGGCCTTCAGCGCGCAGCTGGGG-3') cloned into a pX335 vector containing the Cas9-D10A nuclease sequence

3.2 Construction of donor vector containing the polycistronic reporter cassette

A donor vector containing the following components was designed to be inserted in-frame at the ATG start codon of *ID-1*.

- Complementary DNA (cDNA) sequences encoding for *firefly* luciferase and green fluorescent protein (GFP)

- Two regions (approximately 500 nucleotides) homologous to the *ID-1* gene locus around the start codon, located upstream and downstream of the cassette, to facilitate homologous recombination

- Silent mutations were included in the downstream homologous region to prevent cleavage by the gRNA-directed Cas9 nuclease

- An internal ribosome entry site (IRES) element was incorporated immediately downstream of the luciferase gene to enable separate expression of GFP and luciferase enzyme from the same donor vector

- A sequence encoding the 2A self-cleaving peptide, located downstream of the *GFP* gene, ensures cleavage of the GFP protein from the endogenous ID-1 protein

3.3 Transfection of gRNA and donor vector

Immortalized human keratinocyte HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (subsequently referred to as D10F medium). (**Note:** Conditioned D10F medium was collected from routine HaCaT cultures and filtered for single cell isolation during FACS sorting). Cells were cultured in 10-cm cell culture plates in 9 mL D10F medium and transfected at approximately 60% confluency.

1. Prepare transfection solution by adding 1 µg of sense gRNA, 1 µg anti-sense gRNA, 3 µg cDNA donor vector and 20 µL PEI (1 µg mL^{-1} stock concentration) to 1 mL of sterile Opti-MEM (Reduced Serum Media) in a 15-mL conical centrifuge tube. Vortex for 20 s and incubate at room temperature for 15 min.

2. Add transfection solution drop-wise to the cells, cultured in 9 mL of D10F medium.

3. 24-hours post-transfection, exchange medium for 10 mL fresh D10F medium supplemented with 2 µg mL^{-1} puromycin (**Note**: include an untransfected control cell plate to assess effectiveness of antibiotic selection treatment).

4. 48-hours post-transfection, exchange medium for D10F medium supplemented with 2 µg mL^{-1} puromycin.

5. 72-hours post-transfection, exchange medium for D10F without additional antibiotic and allow the cells to recover for 24 hours (**Optional**: in order to increase the

efficiency of homologous recombination, perform a repeat transfection and antibiotic selection on the same cells following an identical procedure).

6. Allow cells to grow until approximately 90% confluent and prepare them for fluorescence-activated cell sorting (FACS).

3.4 Fluorescence-activated cell sorting (FACS)

1. Aspirate medium and wash cells twice with 1x Dulbecco's Phosphate Buffered Saline (DPBS).

2. Add trypsin (2 mL per 10 cm plate) and incubate at 37°C for 5-10 minutes until the cells detach. Re-suspend cells in 10 mL D10F medium.

3. Transfer cell suspension into a 15 mL conical centrifuge tube and centrifuge at 500 r.p.m. for 3 minutes.

4. Aspirate the supernatant and resuspend the cell pellet in DMEM supplemented with 1% (v/v) FBS at a density of 2.0-5.0 $x10^6$ cells mL^{-1}.

5. Single cells are identified on the basis of FSC-A, FSC-W and SSC-A and GFP fluorescence measured with 488 nm excitation and emission detected at 530 ± 30 nm.

6. Collect individual GFP expressing cells into individual wells of a 96-well cell culture plate containing conditioned D10F medium supplemented with 20% (v/v) FBS (**Note**: centrifuge the 96-well plate at 300 x g for approximately 30 seconds to ensure single cells are at the bottom of the wells).

7. Cells were allowed to proliferate and transferred into progressively larger surface area cell culture plates until sufficient numbers are obtained for subsequent analysis.

3.5 BMP signaling analysis on reporter cells

Single cell clones which were positive for GFP expression and exhibited enhanced luciferase activity compared to control cells were subsequently analysed for responsiveness to BMP stimulation.

1. Seed 1.0 x 10^5 cells per well in a 6-well cell culture plate and culture until approximately 60% confluent.

2. Replace medium with serum-free DMEM for approximately 16 hours (**Note**: serum-deprivation minimizes basal autocrine growth factor/cytokine signalling, thereby enabling the investigation of a single signalling pathway; in this instance the BMP signalling pathway).

3. Prepare the required small molecule inhibitors in serum-free DMEM, in the presence or absence of 6.25 ng mL^{-1} BMP-2, in 15 mL conical centrifuge tubes.

4. The BMP type I receptor (ALK2/3) inhibitor LDN-193189 can used at 100 nM concentration as a positive control for BMP pathway inhibition, whereas the TGFβ type I receptor (ALK4/5/7) inhibitor SB-505124 can be used at 1 µM concentration as a negative control for BMP pathway inhibition.

5. Aspirate serum-free DMEM and dispense 2 mL of each cytokine and/or inhibitor combination into the appropriate well. Incubate for 6-8 hours at 37°C (**Note**: 6-8 hour incubation with 6.25 ng mL^{-1} BMP-2 is sufficient to enable the expression of endogenous ID-1 transcriptional reporter cassette).

3.6 Luciferase Assay

1. Prepare 1x luciferase lysis buffer by adding 1 volume of 5x Cell Culture Lysis Reagent to 4 volumes of ultrapure deionized H_2O (**Note**: equilibrate 1x CCLR to room temperature prior to use).

2. Aspirate media and wash cells twice with 1x DPBS to remove culture medium (**Note**: perform at room temperature).

3. Dispense sufficient volume of 1x CCLR to cover the cells (approximately 300 µL per well in a 6-well cell culture plate) and incubate cells for 5-10 minutes at room temperature with gentle agitation in a shaker.

4. Scrape the cells from the plate surface and transfer lysates into 1.5 mL microcentrifuge tubes on ice.

5. Vortex the cell lysates for 10-15 seconds and then centrifuge at 12,000 x g for 2 minutes at 4°C. Transfer the supernatant into new 1.5 mL microcentrifuge tubes

6. Dispense 40 µL of clarified cell lysate per well of a 96-well microplate (in triplicate for each condition) and subsequently add an equal volume of 2x Luciferase Assay Buffer per well.

7. Seal the microplate with adherent sealing tape and place the microplate on a vibrating platform for 30-60 seconds. Immediately obtain luminescence values using a microplate reader. (**Note**: It is very important to minimise the time between the addition of the luciferase assay buffer and obtaining the luminescence values. Therefore, ensure all equipment and reagents are prepared prior to the addition of the buffer). An example of BMP-dependent luciferase activity using the endogenous BMP reporter cells is included in Figure 1.

8. Use residual cell lysate to determine the protein concentration for each sample to enable normalisation of luminescence values.

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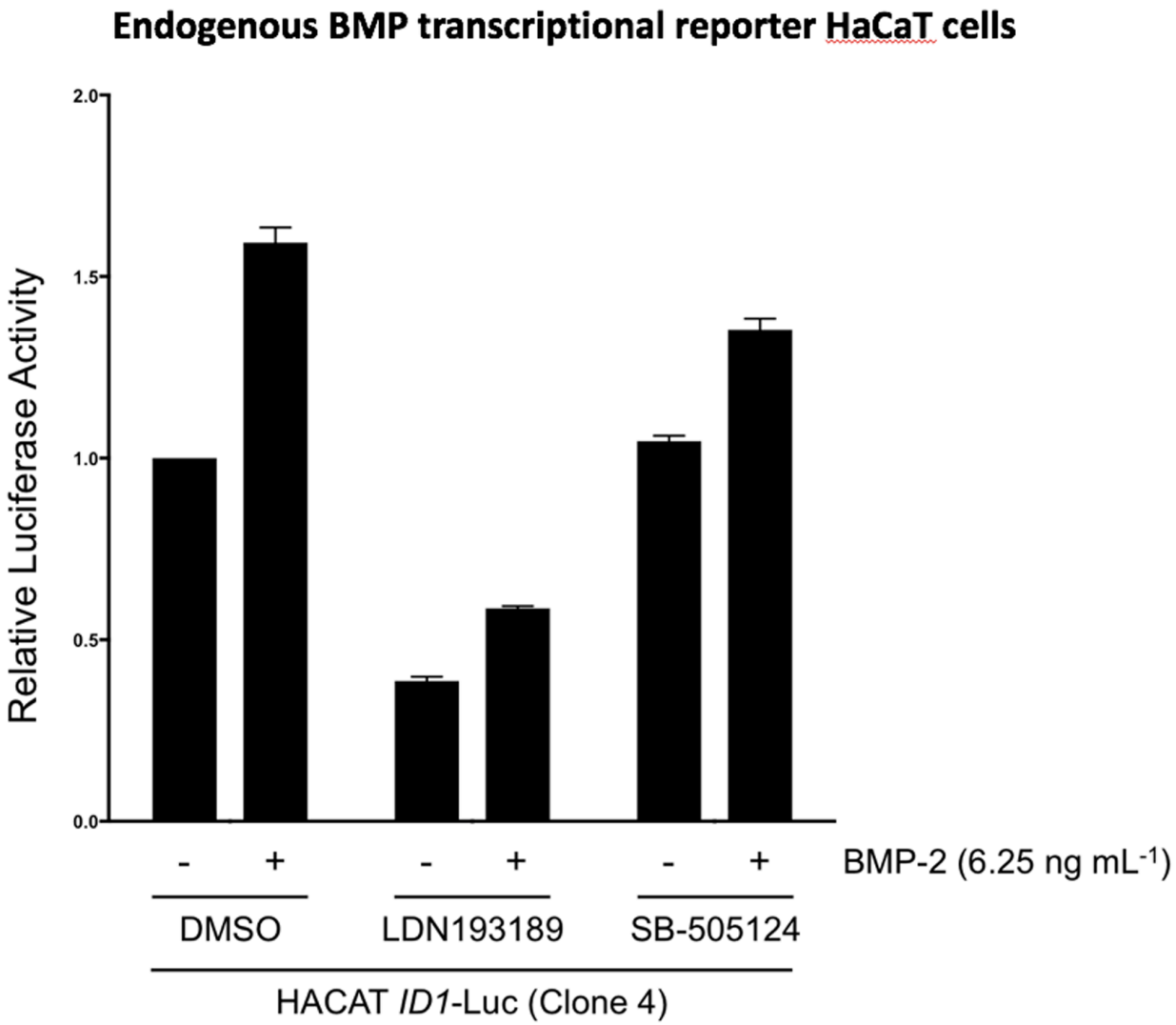
Figure Legend:

Figure 1: Validation of the BMP transcriptional reporter HaCaT cells: GFP-positive single cell isolate (clone 4) was expanded and tested for the insertion of the reporter cassette at the correct locus using BMP-induction and BMPR1 (LDN-193189) and TGFBR1 (SB-505124) inhibitors, as indicated.



Hutchinson et al, Figure 1